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In Vitro Effects of Angiopoietins and VEGF on Hematopoietic and Endothelial Cells

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To investigate the role of TIE-2/angiopoietins (Angs) in postnatal hematopoiesis, we cultured bone marrow cells in the presence of Ang-1 and -2 with or without VEGF. While either Ang-1 or Ang-2 alone had hematopoietic effects on unseparated bone marrow cells and no effect on proliferation of endothelial cells, both enhanced the growth of endothelial cells and hematopoietic progenitor cells in the presence of VEGF. FACS analysis showed that $Lin^-TIE\text{-}2^+Flk\text{-}1^+$ cells cocultured with OP9 stromal cells gave rise to endothelial and hematopoietic cells in the presence of VEGF and Ang-1. Ang-1 promoted the adhesion of sorted primary $Lin^-TIE\text{-}2^+$ cells to fibronectin, and this adhesion enhanced proliferation of hematopoietic progenitor cells synergistically with stem cell factor (SCF). Our findings suggest that TIE-2/angiopoietins may act as critical regulators of proliferation of hematopoietic progenitors and endothelial cells in a synergistic manner. © 1999 Academic Press

Key Words: **hematopoietic progenitors; endothelial cells; cell adhesion and proliferation.**

The development of the hematopoietic cells is closely related to angiogenesis, suggesting the existence of common progenitors, hemangioblasts (1). Endothelial cells are thought to be critical for hematopoiesis, since mice lacking the vascular endothelial growth factor (VEGF) receptor tyrosine kinases Flk-1 and Flt-1, die of defects in endothelial and hematopoietic cell function (2, 3). Furthermore, Flk-1 has been shown to be essential for both endothelial and hematopoietic stem cell development (4). We and other groups have characterized the other subfamilies of endothelial receptor tyrosine kinases (5, 6), TIE-1/TIE and TIE-2/Tek. Mice lacking TIE-2 die from defects in angiogenesis and vascular remodeling, as well as vessel integrity between 9.5 and 10.5 dpc (7, 8). Our previous study also suggested that TIE-2 plays an essential role in the development of definitive hematopoiesis in the aorta-gonad-mesonephros (AGM) region (9), and TIE-2 expressing cells from the AGM region differentiate into hematopoietic and endothelial cells *in vitro* (10).

Mice lacking the ligand for the TIE-2 receptor, Angiopoietin-1 (Ang-1), displays angiogenic deficits similar to but slightly less severe than mice lacking TIE-2 (11). Ang-2 has been reported to act as a natural antagonist for Ang-1, suggesting the presence of an elaborate regulation of the TIE-2/ligand signaling pathway (12). TIE-2 controls the ability of endothelial cells to recruit periendothelial supporting cells to stabilize the structure of blood vessels and modulate their function. However, the precise effects of Angiopoietins on cells expressing TIE-2 have not been elucidated in the adult.

In this study, we analyzed the function of Angiopoietins on TIE-2 expressing cells in bone marrow. We found synergistic effects of Angiopoietins on the proliferation of endothelial cells and hematopoietic progenitors.

MATERIALS AND METHODS

Cell preparation and culture conditions. C57BL/6 mice were purchased from SLC (Shizuoka, Japan). A cell suspension from femur bone marrow (BM) was prepared, and total bone marrow cells or sorted cells were seeded on fibronectin (FN) coated 24-well plates at concentration of 10 μg/ml or on OP9 cells. The culture media were RPMI 1640 (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS) and 50 mM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). The stromal cell line, OP9, was maintained in α-MEM (GIBCO-BRL) supplemented with 20% FCS.

Immunohistochemistry. The procedures for immunohistochemistry were performed essentially as described previously (10). BM cells cultured on FN or OP9 cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and stained with a rat monoclonal anti-mouse platelet endothelial cell adhesion molecule (PECAM-1) antibody (1 μg/ml) (PharMingen, San Diego, CA) by the

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TABLE 1

Colony Formation Activity in Total Primary Bone Marrow Cells after Stimulation by Ang-1,-2 with VEGF

	Nonadherent cells ($\times 10^4$)	No. of colonies/5 $\times$ 10^6 cells			
		G/M/GM	BFU-E	Mix	Total
No factor	12.1 ± 1.8	11.6 ± 3.2	0	0	11.3 ± 3.2
VEGF	13.2 ± 1.5	10.5 ± 3.6	0	0	10.5 ± 3.6
Ang-1	27.7 ± 2.6	27.1 ± 3.6	2.3 ± 0.5	0	28.7 ± 4.5
VEGF + Ang-1	40.6 ± 6.8	30.2 ± 3.3	0	2.5 ± 1.2	32.1 ± 3.8
Ang-2	22.5 ± 2.6	16.2 ± 2.9	0	1.0 ± 0.6	17.0 ± 3.5
VEGF + Ang-2	30.2 ± 4.3	16.5 ± 4.7	0	0	16.8 ± 1.0

Note. 5 $\times$ 10^6 bone marrow cells were cultured on FN-coated culture plates in the presence or absence of different cytokines. The doses applied were as follows: Ang-1, 300 ng/ml; Ang-2, 300 ng/ml; and VEGF, 10 ng/ml. After 7 days of culture, cells were harvested, counted, and transferred to methyl-cellulose medium containing IL-3, SCF, and Epo. The number of colonies (>40 cells) was scored after 7 days of culture. The results represent triplicate samples.

indirect immunoperoxidase method using horseradish peroxidase-conjugated anti-rat IgG (9).

Flow cytometric analysis and cell sorting. Cell suspensions were stained for 30 min with the following monoclonal antibodies (MoAbs): anti-TIE-2 (TEK4) was previously prepared in our laboratory; Sca-1 (E13-161.7), Gr-1 (RB6-8C5), Mac-1 (M1/70), TER119, B220 (RA3-6B2), CD4 (RM4-5), and CD8 (53-6.70), all of which were purchased from PharMingen; Anti-c-Kit (ACK2, rat anti-mouse MoAb) (13) and anti-Flk-1 antibody (AVAS12, rat anti-mouse MoAb) (14) were gifts from Dr. S.-I. Nishikawa, Kyoto University, Kyoto, Japan. All MoAbs were purified and conjugated with PE, FITC or biotin. Biotinylated antibodies were visualized with PE-conjugated streptavidin (GIBCO-BRL) or FITC-conjugated streptavidin (Sigma). A mixture of Gr-1, Mac-1, TER119, B220, CD4, and CD8 was used as lineage (Lin) markers. Fluorescence-activated cell sorting (FACS) was performed on a FACSvantage (Becton–Dickinson Immunocytometry Systems, San Jose, CA).

Progenitor assay by methylcellulose culture. Cultured hematopoietic cells were embedded in 1 ml of α-medium containing 1.3% methylcellulose (1500 cp; Aldrich Chemical Co., Milwaukee, WI), 30% FCS, 1% deionized bovine serum albumin (BSA) (Sigma), 0.1 mmol/mL 2-ME, 100 ng/mL stem cell factor (SCF) (from Chemo-Sero-Therapeutic Co. Ltd., Kumamoto, Japan), 100 U/mL recombinant mouse IL-3, 20 ng/ml human recombinant IL-6 (provided by Ajinomoto, Kawasaki, Japan), and 2 U/ml recombinant human erythropoietin (EPO) (provided by Snow-Brand Milk Product Co. Ltd., Tochigi, Japan). The cells were cultured in a 35-mm culture dish and incubated at 37°C in a humidified atmosphere with 5% CO_2. On the seventh day of culturing, aggregates containing of 40 or more cells were counted as a single colony.

Adhesion of primary TIE-2$^+$ cells. Primary TIE-2$^+$ cells (10^4) were sorted by FACSvantage and incubated in the presence or absence of human recombinant Ang-1 (50–300 ng/mL) with or without TIE-2-Fc or GRGDS peptides (0.5 mg/ml, American Peptide Co., Sunnyvale, CA) for 30 min at 37°C. TIE-2-Fc was a recombinant fusion protein of the extracellular domain of murine TIE-2 and the Fc portion of human Ig (100-fold molecular weight of Ang-1). Before applying the cells to the plate, both FN-coated and noncoated plates

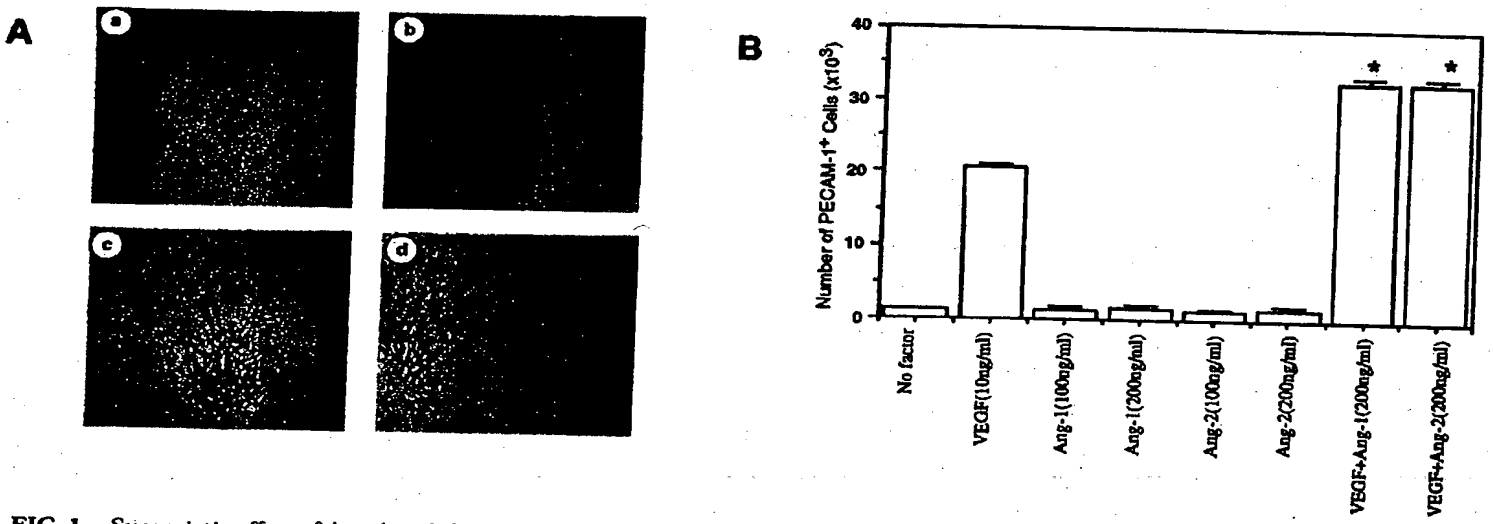

FIG. 1. Synergistic effect of Ang-1 and -2 with VEGF on proliferation of endothelial cells. (A) Adherent PECAM-1$^+$ endothelial cells were visualized after culture of unseparated BM on FN for 10 days in the presence of (a) Ang-1 (300 ng/ml), (b) VEGF (10 ng/ml), (c) Ang-1 (300 ng/ml) + VEGF (10 ng/ml), and (d) Ang-2 (300 ng/ml) + VEGF (10 ng/ml). (B) Quantification of PECAM-1$^+$ cells grown with various factors. Plates were cultured for 10 days and then washed to remove suspension cells. The adherent layers were digested by Dispase and stained with PECAM-1 MAb. The frequency of PECAM-1$^+$ cells was evaluated by FACS in triplicate wells (mean ± SD). Note: * indicates a significant difference from VEGF alone (10 ng/ml) ($p < 0.01$).

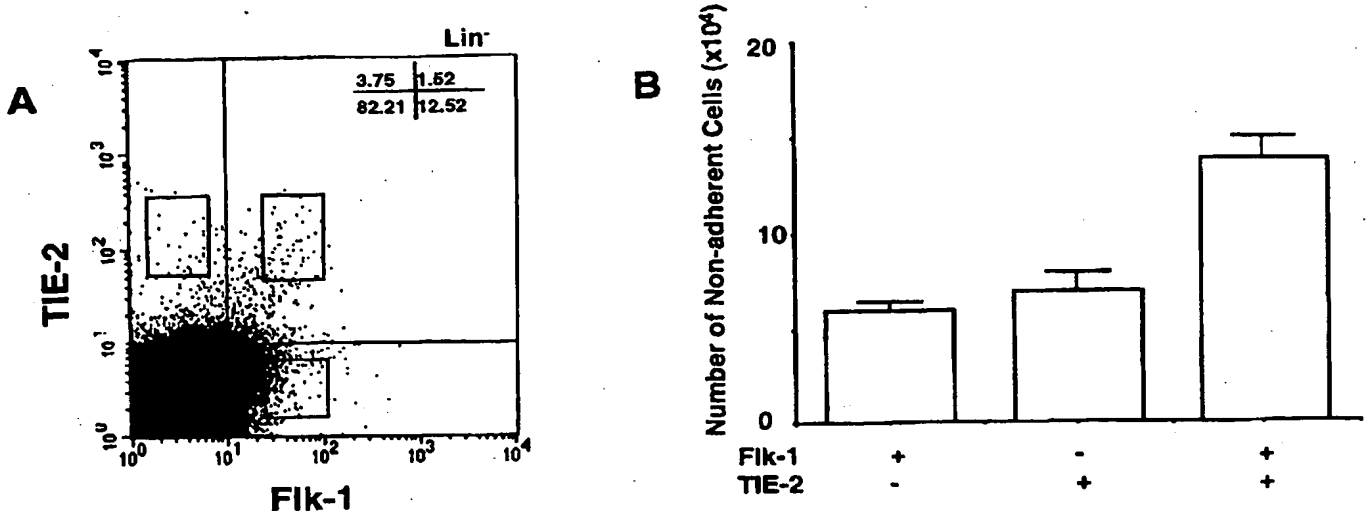

FIG. 2. Hematopoietic cell development from sorted fractions of BM cells. (A) The cells disaggregated from BM were stained with anti-TIE-2 MAb, Flk-1 MAb and Lin mix MAbs, and then fractionated by FACS. Sorting gate is indicated by the small box. The percentage in each quadrant is representative of triplicate experiments. (B) Proliferation of 10^4 sorted hematopoietic cells; $Lin^-Flk\text{-}1^+TIE\text{-}2^-$, $Lin^-Flk\text{-}1^-TIE\text{-}2^+$, and $Lin^-Flk\text{-}1^+TIE\text{-}2^+$ cells. After culture on OP9 cells in the presence of VEGF and Ang-1 for 10 days, nonadherent cells were collected and counted.

were preincubated with 2% BSA in PBS. To determine adherence to FN, floating cells were harvested by several rounds of gentle pipetting before the remaining cells were counted.

RESULTS

Synergistic effects of VEGF and angiopoietins on proliferation of hematopoietic and endothelial cells. We first examined the effects of Ang-1, -2, and VEGF on unseparated BM cells. While VEGF did not show hematopoietic effects, Ang-1 and Ang-2 promoted proliferation of hematopoietic cells and colony-forming cells (CFU-Cs) (Table 1). These effects were significantly enhanced when Ang-1 and Ang-2 were combined with VEGF. Cytospin analyses revealed that proliferating cells were mainly granulocytes and macrophages. FACS analyses showed that $Lin^-c\text{-}Kit^+$ hematopoietic

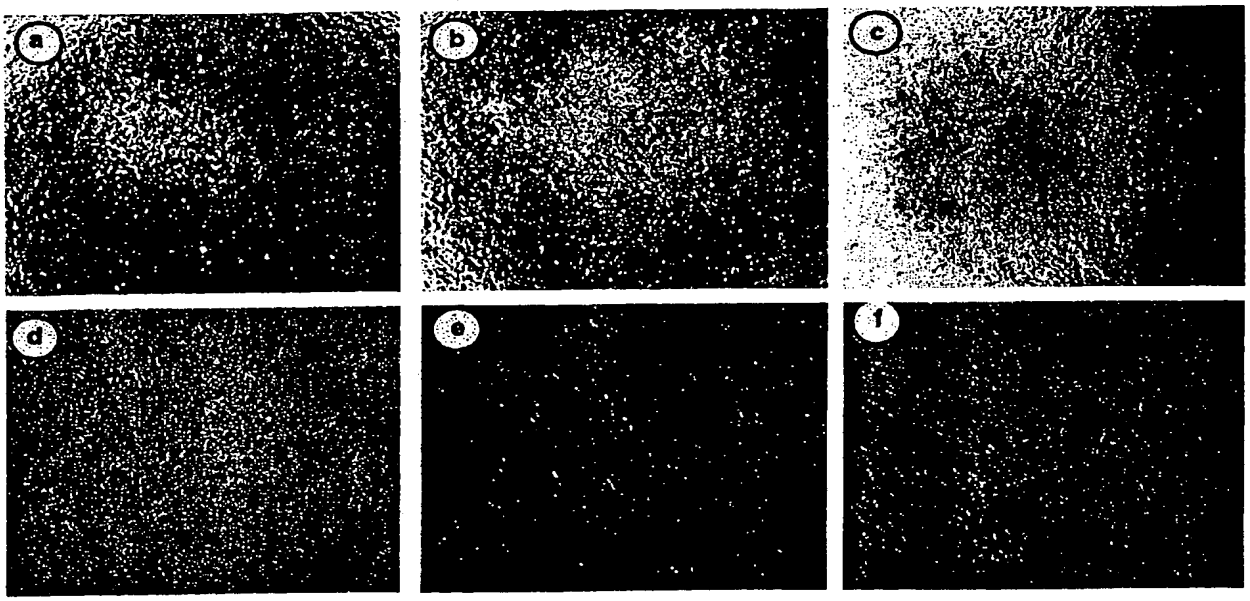

FIG. 3. Hematopoietic and endothelial cell development from sorted fractions of BM cells. Sorted 10^4 hematopoietic cells; $Lin^-Flk\text{-}1^+TIE\text{-}2^-$ (a, d), $Lin^-Flk\text{-}1^-TIE\text{-}2^+$ (b, e) and $Lin^-Flk\text{-}1^+TIE\text{-}2^+$ (c, f) cells were cultured on OP9 feeder cells in the presence of VEGF and Ang-1. The growth of hematopoietic cells was seen in three fractions after 10 days of culture (a, b, and c). A vascular network of PECAM-1^+ endothelial cells was observed only in the $Lin^-Flk\text{-}1^+TIE\text{-}2^+$ fraction (f).

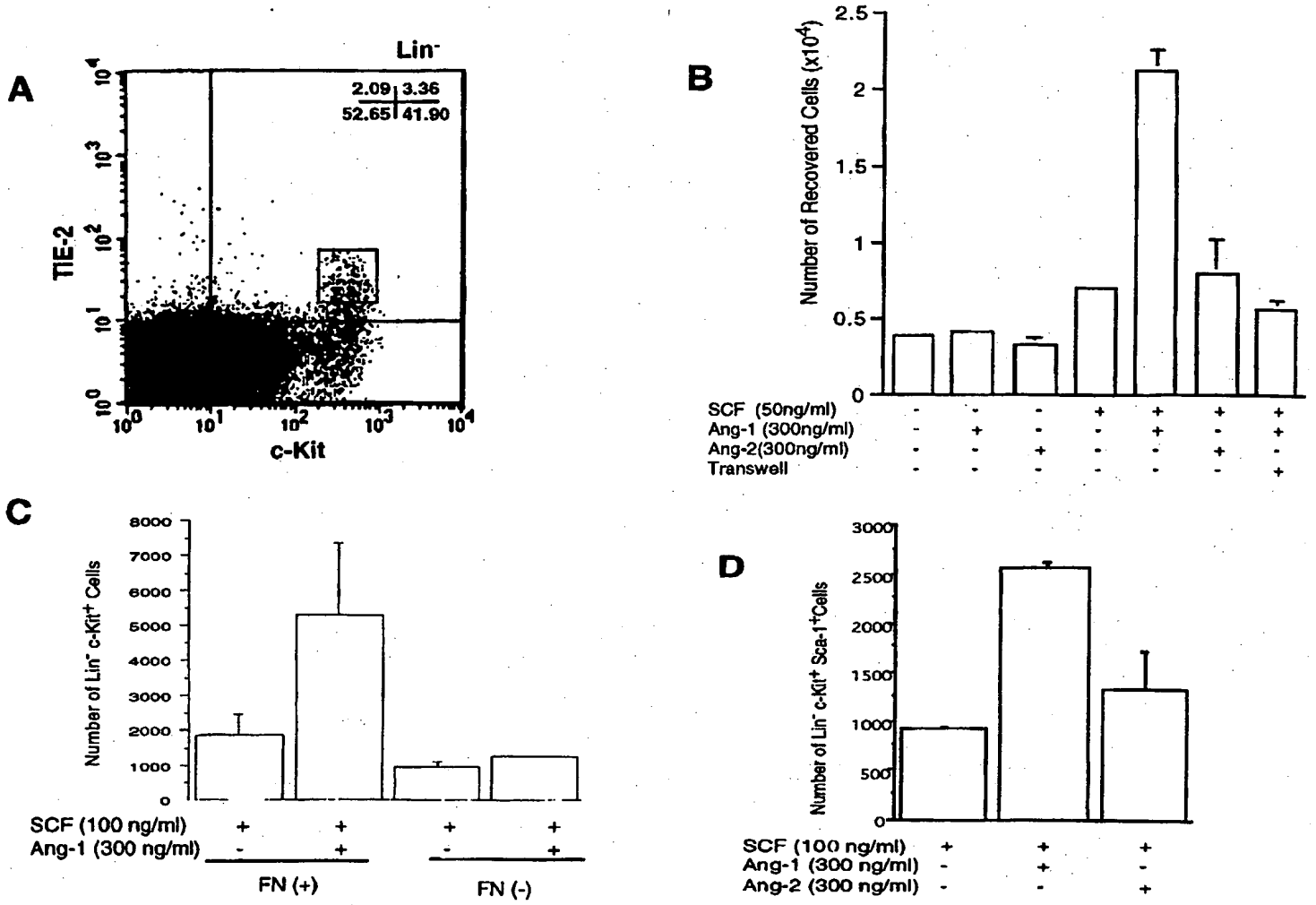

FIG. 4. Synergistic effect of Ang-1 and SCF on proliferation of primary Lin⁻c-Kit⁺TIE-2⁺ cells. (A) Cells disaggregated from total BM were stained with anti-TIE-2 MoAb, c-Kit MoAb and Lin mix MoAb, and then fractionated by FACS. Sorting gate is indicated by the small box. The percentage in each quadrant is representative of triplicate experiments. (B) Two thousand primary Lin⁻c-Kit⁺TIE-2⁺ cells sorted from BM were cultured on FN-coated culture plates in the presence or absence of different cytokines. After 7 days of culture, all recovered cells were harvested and scored. (C) Expansion of Lin⁻c-Kit⁺ cells. Primary Lin⁻c-Kit⁺TIE-2⁺ cells were seeded on FN plates or uncoated plates (FN-) and cultured for 7 days. Recovered cells were collected and counted. The frequency of Lin⁻c-Kit⁺ cells was evaluated by FACS in triplicate wells (mean ± SD). (D) Expansion of Lin⁻c-Kit⁺Sca-1⁺ cells. Primary Lin⁻c-Kit⁺TIE-2⁺ cells were seeded on FN plates or uncoated plates and cultured for 7 days. Recovered cells were collected and counted. The frequency of Lin⁻c-Kit⁺Sca-1⁺ cells was evaluated by FACS in triplicate wells (mean ± SD).

progenitor cells increased in number (data not shown), which is consistent with the increased number of CFU-Cs. On the other hand, Ang-1 and -2 alone had no effects on cell growth but acted synergistically with VEGF to stimulate growth of PECAM-1⁺ endothelial cells (Figs. 1A and 1B).

Differentiation of hematopoietic and endothelial cells from the sorted Lin⁻TIE-2⁺Flk-1⁺ fraction. VEGF and either Ang-1 or Ang-2 synergistically stimulate proliferation of hematopoietic and endothelial cells. To examine the expression of TIE-2 (a receptor for Angiopoietins) and Flk-1 (a receptor for VEGF) in BM by FACS (Fig. 2A) and found that 30% of Lin⁻TIE-2⁺ cells coexpressed Flk-1, which constituted 0.015% of the total BM cells from 3-month-old mice. Lin⁻TIE-2⁺Flk-1⁺ cells generated PECAM-1⁺ endothelial cells and hematopoietic progenitors in the presence of Ang-1 and VEGF co-cultured with OP9 cells for 14 days, while Lin⁻TIE-2⁺Flk-1⁻ cells or Lin⁻TIE-2⁻Flk-1⁺ cells generated fewer nonadherent hematopoietic cells (Fig. 2B). Moreover, Lin⁻TIE-2⁺Flk-1⁻ cells or Lin⁻TIE-2⁻Flk-1⁺ cells did not give rise to PECAM-1⁺ cells (Fig. 3).

Stem cell proliferation of primary Lin⁻c-Kit⁺TIE-2⁺ cells in the presence of Ang-1 and SCF. To examine the effect of Ang-1 and -2 on hematopoiesis, hemato-

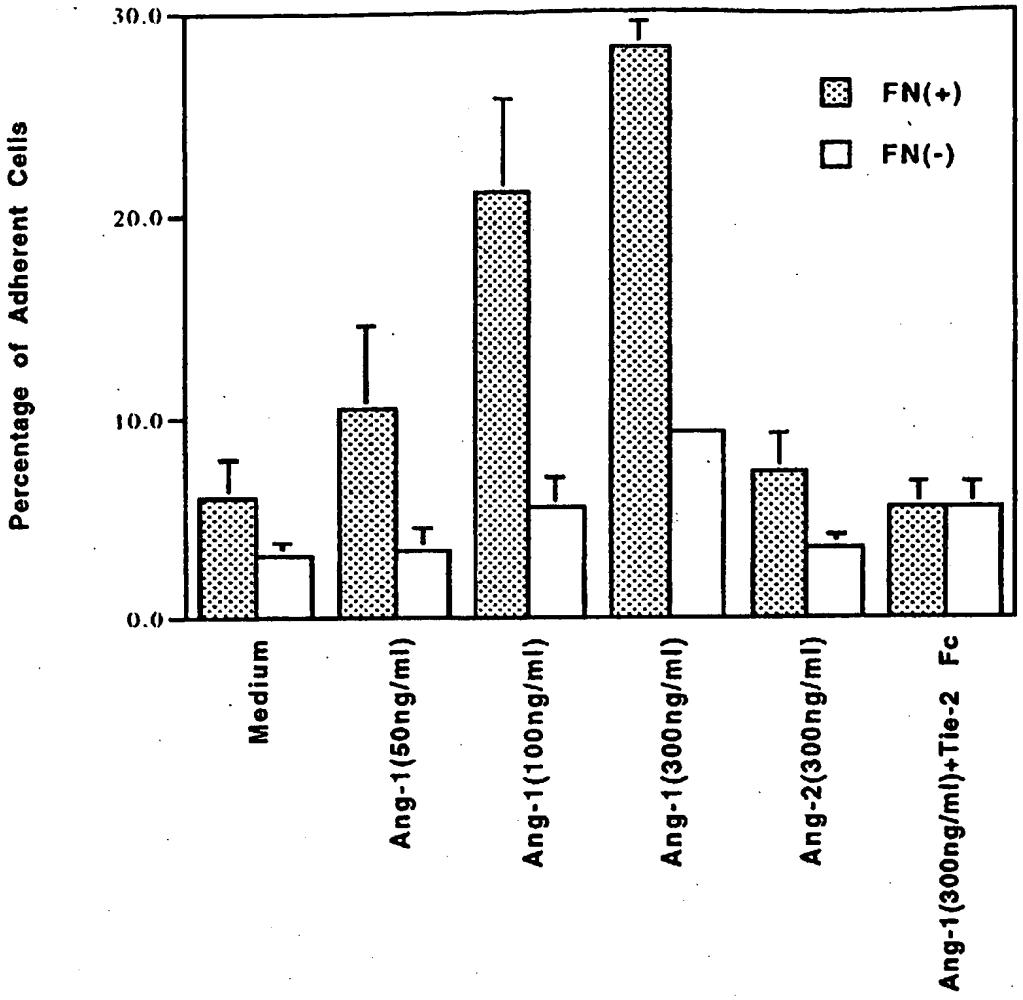

FIG. 5. Adhesion of TIE-2$^+$ hematopoietic cells to FN following stimulation by Ang-1. Sorted Lin$^-$TIE-2$^+$ cells adhered to FN-coated plates following Ang-1 treatment in a dose-dependent manner. This adhesion was blocked by saturation of ligands with TIE-2-Fc or by a GRGDS peptide. Error bars indicate standard error of the mean (n = 3).

poietic progenitor cells or Lin$^-$c-Kit$^+$TIE-2$^+$ cells were sorted (Fig. 4A) by FACS and cultured on FN-coated or non-coated plates in the presence of Angiopoietins and/or SCF (a ligand for c-Kit receptor). Ang-1 combined with SCF enhanced cell proliferation more effectively than SCF alone. Hematopoietic cells and CFU-Cs grown in each condition were examined (Figs. 4B, 4C, and 4D). It is evident that Ang-1 enhanced proliferation of hemotopoietic progenitor cells synergistically with SCF on FN-coated plates. This effect was neither observed, when we applied a Transwell apparatus, which inhibits contact of hematopoietic cells to FN (Fig. 4B) nor on noncoated plates (Figs. 4C and 4D). It suggested that direct contact of Lin$^-$c-Kit$^+$TIE-2$^+$ cells to FN was required for hematopoietic cell growth. In all, the enhanced proliferation of Lin$^-$c-Kit$^+$cells and Lin$^-$c-Kit$^+$Sca-1$^+$ cells by Ang-1 with SCF was about 2.5 times higher than in the presence of SCF alone (Fig. 4D).

Adhesion of Lin$^-$ TIE-2$^+$ hematopoietic cells to FN. Next we sorted primary Lin$^-$TIE-2$^+$ cells from adult BM and determined the proportion of adherent cells in order to analyze the effect of Ang-1. Cell adhesion to FN was promoted by Ang-1 in a dose-dependent manner, but not by Ang-2 (Fig. 5). TIE-2-Fc inhibited Ang-1 mediated adhesion to FN to basal levels. Integrin GRGDS peptide completely suppressed Ang-1-mediated adhesion to FN. Thus, adhesion to FN may be mediated by integrins through the GRGDS motif.

DISCUSSION

In this report, we show the *in vitro* synergistic effects of Ang-1 and -2 and VEGF on TIE-2 expressing cells in BM. It has been shown that Ang-1 does not affect endothelial cell growth in culture (15), but that mice lacking Ang-1 display angiogenic deficits similar to but slightly less severe than those lacking TIE-2 (11).

Ang-1 and Ang-2 enhance VEGF-induced angiogenesis *in vivo* in the corneal micropocket assay (16). It was also reported that Ang-1 increases sprouting and branching when combined with VEGF, suggesting that Ang-1 and VEGF have distinct and complementary roles (17, 18). We show that Ang-1 is not as effective as VEGF in promoting proliferation of endothelial cells in culture. Taken together, VEGF is the main mitogenic factor of endothelial cells and is crucial for embryonic vasculogenesis and angiogenesis; however, Angiopoietins act synergistically with VEGF. A weak effect of Angiopoietins was detected on unfractionated bone marrow cells (Table 1) but not on a stem cell fraction (Fig. 4B). Hematopoietic cell growth may be enhanced by the endothelial cell proliferation in the presence of Angiopoietins and VEGF. We show that TIE-2$^+$Flk-1$^+$ cells co-cultured with OP9 cells generated both endothelial and hematopoietic cells in the presence of Ang-1 and VEGF. It remains to be clarified whether common progenitors such as hemangioblasts give rise to hematopoietic cells and endothelial cells simultaneously or progenitors of hematopoietic cells and endothelial cells were discriminated from each other.

Angiopoietins alone is neither mitogenic nor anti-apoptotic to purified hematopoietic progenitors. We show that Ang-1 but not Ang-2 promotes adhesion to FN in adult Lin$^-$TIE-2$^+$ BM cells. Cell adhesion is mediated by the interaction between integrins on TIE-2$^+$ hematopoietic stem cells stimulated with Ang-1 and FN on endothelial cells. Ang-1 induced adhesion of Lin$^-$TIE-2$^+$ cells to FN and hematopoietic proliferation was subsequently enhanced in the presence of SCF. This suggests that cell adhesion induced by TIE-2 and its ligand, Ang-1 is important for the proliferation of primitive hematopoietic cells. It is controversial whether Ang-2 is antagonistic to the Ang-1 signaling via TIE-2 receptor (12). There is evidence that not only Ang-1 but Ang-2 promote the phosphorylation of TIE-2 in non-endothelial cells including hematopoietic cells (12, 19). Differing from Ang-1, Ang-2 induced the aggregation of TIE-2-positive cells, resulting the reduction in cell adhesion to FN.

In conclusion, our findings strongly support the idea that the signals of TIE-2/Ang-1 not only enhance the mitogenic effect of VEGF on growth of endothelial cells but is also critical for regulation of the proliferation of hematopoietic progenitor cells coordinated with SCF.

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